D7C

(2)

National Défense
Defence nationale

UNCLASSIFIED

AD-A257 092

UNLIMITED
DISTRIBUTION

DRES

SUFFIELD MEMORANDUM

NO. 1383

PURIFICATION AND PARTIAL CHARACTERIZATION OF MONOCLONAL ANTIBODIES OF *Francisella tularensis*

by

DTIC
ELECTE
NOV03 1992
S D
E

A.R. Bhatti, J.P. Wong and Donald E. Woods

July 1992

92-28700

DEFENCE RESEARCH ESTABLISHMENT SUFFIELD, RALSTON, ALBERTA

DEFENSIO PER SCIENTIAM

Canada

WARNING
"The use of this information is permitted subject to recognition of proprietary and patent rights".

92 11 104

DEFENCE RESEARCH ESTABLISHMENT SUFFIELD

RALSTON, ALBERTA

SUFFIELD MEMORAMDUM NO. 1383

PURIFICATION AND PARTIAL CHARACTERIZATION OF MONOCLONAL ANTIBODIES OF *Francisella tularensis*

A.R. Bhatti, J.P. Wong and Donald E. Woods

WARNING
"The use of this information is permitted subject to recognition of proprietary and patent rights".

Toxicology and Infectivity Group
Defence Research Establishment Suffield,
Ralston, Alberta TOJ 2NO Canada
Department of Microbiology and Infectious Diseases Faculty of Medicine, University of Calgary Science Centre
Calgary, Alberta T2N 4N1 and

DTIC QUALITY INSPECTED 1

Accesion For
NTIS CRA&I
DTIC TAB
Unannounced
Justification
By
Distribution /
Availability Codes
Dist | Avail and / or Special
A-1

ABSTRACT

Hybridomas secreting monoclonal antibodies against *Francisella tularensis* cellular antigens were isolated. These monoclonal antibodies reacted with killed whole cells of *F. tularensis* in the ELISA but not by immunoblot, indicating that the antibodies were directed against conformational epitopes. One of these monoclonal antibodies was directed principally against outer membrane protein (OMP) components, and the remainder are likely directed against capsular components. The OMP-specific monoclonal antibodies are *F. tularensis*-specific in contrast to the others which cross-react with a number of other bacterial species. These OMP-specific monoclonal antibodies are of the IgG_1 subclass, and may prove to be a useful tool for diagnosis of *F. tularensis* in clinical materials.

RÉSUMÉ

Des hybridomes sécrétant des anticorps monoclonaux dirigés contre des antigènes cellulaires de *Franciselle tularensis* ont été isolés. Ces anticorps monoclonaux ont réagi avec des cellules entières tuées de *F. tularensis* dans un test ELISA, mais non dans le test <<immunoblot>>, ce qui indique que ces anticorps sont dirigés contre des épitopes conformationnels. Un de ces anticorps monoclonaux était dirigé principalement contre des constituants d'une protéine de la membrane externe (PME) et les autres étaient vraisemblablement dirigés contre des constituants de la capsule. Les anticorps monoclonaux spécifiques de la PME sont spécifiques de *F. tularensis*, contrairement aux autres qui donnent une réaction croisée avec un certains nombre d'autres espèces bactériennes. Ces anticorps monoclonaux spécifiques de la PME appartiennent à la sous-classe des IgG_1 et pourraient se révéler un outil précieux pour l'identification, à des fins diagnostiques, de *F. tularensis* dans des prélèvements cliniques.

INTRODUCTION

Francisella tularensis is found primarily in wild mammals and blood-sucking arthropods (Francis, 1928). *F. tularensis* infection in humans is termed tularemia and is characterized by high fever and severe symptoms that may persist for weeks to several months if not treated appropriately (Sanders and Hahn, 1968; Evans *et al*, 1985). Although the reported incidence of human tularemia in the North America has declined dramatically in the last 3 decades, the disease is still responsible for appreciable morbidity in some parts of the world and among certain occupational groups (Boyce, 1975).

F. tularensis is difficult to visualize in Gram stains of clinical specimens such as sputum or exudate from skin ulcers or affected lymph nodes. Cultures are usually negative since the organism does not grow on most ordinary media (Berdal and Soderlund 1977). Laboratories are reluctant to attempt, isolation of *F. tularensis* because of the risk of creating infectious aerosols in the laboratory. This is a significant worry as, in humans, 10-50 organisms will cause disease if inhaled.

The organism grows optimally at 37°C under aerobic conditions, with small, smooth, opaque colonies appearing in 24-48 h. It is usually identified on the basis of its morphologic characteristics, growth requirements, fluorescent staining and agglutination with specific antisera (Owen, 1970). *F. tularensis* possesses a

number of cell-associated antigenic components, including a polysaccharide capsular antigen, a protein antigen that cross-reacts with *Brucella* and an endotoxin-like substance with biologic activity similar to endotoxins produced by other gram-negative bacteria (Francis and Evans, 1926). The objective of the present study was to produce, purify and characterize monoclonal antibodies against cell-surface antigens of *F. tularensis* for the purpose of employing these in diagnosis of infection due to this organism.

MATERIALS AND METHODS

Bacterial Strain. Clinical isolates of *tularensis*, *Pasteurella multocida*, *Escherichia coli*, *Hemophilus influenzae*, *Klebsiella pneumoniae*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, and *Brucella abortus* were obtained from the Clinical Microbiology Laboratory at Foothills Hospital, Calgary, Alberta. Organisms were grown on enriched agar plate media (charcoal-yeast-extract) for 24 h at 37°C, the growth scraped from the surface and resuspended in phosphate buffered saline (PBS, 0.05 M, pH 7.2).

Antigen Preparation. *F. tularensis* LVS whole cells were prepared for immunization by culturing in a chemically defined synthetic medium (Chamberlain 1965). For preparation of formalinized cells, cultures were centrifuged at 15,380 g for 20 min at 4°C in a J2-21 centrifuge (Beckman Instruments, Fullerton, CA) and each pellet was suspended in 100 ml of physiological saline. Formaldehyde solution was then added to achieve a final concentration of 2.4%. The formalinized culture was held at 4°C with slight agitation for 48 h, then centrifuged at 15,380 x g for 20 min at 4°C. The resulting pellet was resuspended in 100 mL of fresh 2.4% formalin-saline and incubated with constant stirring at 4°C for 1 wk. For viability, test aliquots of formalinized bacterial suspensions were inoculated on plates containing synthetic medium supplemented with 1.5% agar and incubated

for 4 days at 37°C in 5% CO_2 air atmosphere. No colonies of *F. tularensis* were detected. Prior to the ELISA, bacterial suspensions were washed several times in PBS to remove residual formalin and the viability check was repeated. Outer membrane protein preparations of *F. tularensis* were obtained according to the published procedures (Ormsbee *et al*, 1954; Prochzaka and Dubanska, 1972). Hyperimmune ascites fluids against *F. tularensis* LVS OMP and whole cells were produced using method described (Brandt *et al*, 1967; Russel *et al*, 1970; Chiewslip and McCowan, 1972).

Monoclonal Antibody Preparation. Dulbecco modified Eagle medium (DMEM) with sodium pyruvate (Flow Laboratories, Mississauga, ON), was supplemented with glutamine, penicillin, streptomycin and 10% fetal calf serum (Myoclone; Gibco, Burlington, ON). Hypoxanthine-aminopterin-thymidine and hypoxanthine-thymidine media were obtained from Flow Laboratories. The myeloma cell line NS-1 was used to prepare hybridomas. These cells were maintained in DMEM-10% fetal calf serum. When the cell density reached approximately 10^7 cells per ml, cells were diluted to 1 X 10^6 to 2 X 10^6 cells per ml in fresh DMEM-10% fetal calf serum and transferred. Cell cultures were maintained at 37°C under 7% CO_2.

Hybrid cell lines were prepared by published methods (Oi and Herzenberg, 1980). BALB/c mice were injected four times intraperitone-

ally with 50 µg of antigen on days 0, 7, 14 and 21. Three days after the final injection, spleens were removed, and the spleen cells were collected. Spleen and NS-1 cells (10:1) were fused by 50% (w/w) polyethylene glycol. Fused cells were transferred into microtiter dishes at 6 X 10^4 input spleen cells per well and maintained for 14 days in hypoxanthine-amopterin-thymidine medium. Hybridoma cell lines producing monoclonal antibody to *F. tularensis* antigens detectable by ELISA were transferred to hypoxanthine-thymidine medium and cloned by limiting dilution. Antibody-producing cells (2 X 10^6) were injected into pristane-primed mice for ascites tumor induction.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed on 12.5% acrylamide- 0.17% bis (acrylamide)-SDS slab gels (Laemmli, 1970).

Electrophoretic blotting procedure. Whole cells and outer membrane preparations of *F. tularensis* were electrophoresed as described above and transferred to nitrocellulose with a Bio-Rad Richmond, CA. transblot apparatus for 30 min at 1 A. The nitrocellulose was incubated at 37°C for 1 h in 0.05% Tween 20 in PBS to block nonspecific binding of antibodies. The nitrocellulose paper was incubated for 2 h at 37°C with ascitic fluid at a dilution of 1:1,000. The blots were then incubated with horseradish peroxidase-conjugated protein-A from *Staphylococcus aureus* (Sigma, St. Louis, MO.) at a 1:2,000 dilution in PBS containing 0.05% Tween 20

for 2 h at 37°C. Colour was developed with Bio-Rad HRP colour reagent as recommended by the manufacturer (Bio-Rad, Richmond, CA).

Subisotyping of monoclonal antibodies. The subisotype of antibodies in the ascitic fluid was determined by using a mouse antibody subisotyping kit (Bio-Rad Mississauga, ON.) as recommended by the manufacturer.

ELISA. Fifty µg of whole-cell protein equivalent of bacterial strains were fixed with ethanol onto microtiter U-well plates (Dynatech Laboratories, Inc., Alexandria, VA). The plates were blocked with 1% bovine serum albumin in PBS for 1 h at 37°C. Wells were washed with 0.05% Tween 20-PBS three times. Fifty µl of monoclonal antibody were added per well, and the plates were incubated for 2 h at 37°C. Plates were washed with 0.05% Tween 20-PBS three times. Fifty µl of a 1:2500 dilution of goat anti-mouse IgG and IgM labeled with horseradish peroxidase (Kirkegaard and Perry Laboratory, Inc., Gaithersburg, MD) were added, and the plates were incubated for 2 h at 37°C. Plates were washed with 0.05% Tween 20-PBS three times. Fifty µl of peroxidase substrate (Kirkegaard and Perry) were added, and the plates were allowed to incubate for 15 min before they were read (MR580 MicroElisa AutoReader, Dynatech).

Antibody Purification. Antibodies were purified by a column chromatography procedure on hydroxyapatite (Bukovsky and Kennett, 1987). A

column of hydroxyapatite (Bio-Rad grade HT) was prepared by resuspending the fully hydrated material in 10 mM sodium phosphate buffer pH 6.8. The column was washed with 10 column volumes of the same sodium phosphate buffer. Approximately 100 ml of hydroxyapatite was used to bind the antibody from 500 ml of tissue culture supernatant or from 5 ml of ascites. The antibody solutions were centrifuged at 4,000 X g for 15 min prior to loading onto the column. Ascites fluid was diluted 1:10 with distilled water prior to centrifugation. The sample was passed down the column and the column washed with 20 column volumes of 10 mM sodium phosphate buffer pH 6.8. The antibodies were eluted by raising the phosphate concentration by applying a linear gradient of 120-300 mM sodium phosphate buffer pH 6.8. Fraction (5 ml) were collected at a flow rate of 10 ml/h. Fraction containing antibodies were pooled, dialyzed against 10 mM ammonium bicarbonate for 24 h, lyophilized and stored at -20°C.

RESULTS

Isolation of monoclonal antibodies against *F. tularensis*. Ten hybridomas producing monoclonal antibodies against *F. tularensis* were isolated from a fusion of NS-1 cells and spleen cells from mice immunized with *F. tularensis* (OMP or whole cells). Positive clones were detected by using an ELISA with *F. tularensis* whole cells and OMP as antigen. Four of the positive hybridoma cell lines were single-cell cloned and injected into pristane-primed BALB/c mice for ascites tumor induction. The ascitic fluid from each of the injected hybridomas was shown to react with *F. tularensis* in ELISA as well as against other bacterial species (Table 1). Hybridoma 179.4 is the only hybridoma with specificity for *F. tularensis*. Based on the cross-reactions with other bacterial species, it seems clear that 179.4 is a *F. tularensis* specific monoclonal antibody producer (Table 1).

Classification of monoclonal antibodies. The monoclonal antibodies were classified in terms of immunoglobulin subclass (Table 2) and ELISA titer against *F. tularensis* outer membrane preparations and whole cells (Table 3). One of the monoclonal antibodies was of the IgG1 subclass (179.4) while the remaining three monoclonal antibodies (F47-6, F30-20, F47-2) reacted with antisera against IgG1 and IgG3 subclass antigens. Hybridoma 179.4 is most likely directed against an outer membrane component. The titer against outer membrane preparation

is higher than that against whole cell preparations (where the capsule is the most likely antigen source). As repeated attempts (>5X) to demonstrate by Western blots that 179.4 reacts with an outer membrane protein were unsuccessful, we have concluded that the monoclonal antibodies are directed against a conformational epitope which is lost upon SDS-PAGE and electrophoretic transfer to nitrocellulose paper.

Purification of monoclonal antibodies. Repeated (>3X) attempts to obtain ascites fluid upon intraperitoneal injection of 179.4 hybridoma cells into pristane-primed mice did not meet with success; thus, monoclonal antibodies were purified from tissue culture fluids by hydroxylapatite chromatography. SDS-Page of purified antibodies did not show any detectable contaminating protein (data not shown).

DISCUSSION

The present studies were designed to develop monoclonal antibodies against cellular components of *Francisella tularensis* for the purposes of employing them as diagnostic reagents and/or for use in structure-function studies. Those monoclonal antibodies obtained from hybridoma 179.4 could prove to be particularly useful as diagnostic probes for the detection and identification of *F. tularensis* due to their relative specificity for this organism. These antibodies are directed against a protein antigen located in the outer membrane fraction of *F. tularensis*, and the specificity as demonstrated by ELISA appears to be adequate for differentiation of *F. tularensis* from the other bacterial organisms examined. Thus, these antibodies could be employed to detect *F. tularensis* directly in clinical specimens; or, alternatively, it could be employed in a culture-amplified detection system such as has been developed for *Mycoplasma pneumoniae* (Cimolai, *et al*, 1988).

It was interesting to note that those monoclonal antibodies, other than 179.4, appear to be directed against whole cell surface antigens which are likely polysaccharide in nature. Further, these antibodies do not specifically react with *F. tularensis*. Similar cross-reactivity has been observed for polyclonal antibody against *B. abortus* (Cherwonogrodzky *et al*, 1990). While these antibodies do not appear to be useful as diagnostic reagents, they may certainly prove to be useful in probing "common"

antigens or epitopes which appear to be associated with a variety of bacterial organisms examined. It is not clear as to why these whole cell surface antigens appear to be common to a number of bacterial species; however, the use of the monoclonal antibodies developed in these studies could be very useful in dissecting the answer to this question.

TABLE 1

ANTIBODY TITERS* TO DIFFERENT BACTERIAL SPECIES

HYBRIDOMA

ORGANISM	F47-6	179.4	F30-20	F47-2
P. multocida	163840	2560	81920	10240
E. coli	10240	2560	5120	1280
H. influenzae	1280	1280	1280	320
K. pneumoniae	80	10	1280	320
Y. enterocolitica	2560	1280	2560	320
P. aeruginosa	640	1280	2560	1280
L. pneumophila	80	40	320	10
B. abortus	80	80	2560	320
F. tularensis	2560	10240	5120	2560

* Antibody Titer by ELISA

TABLE 2

IMMUNOGLOBULIN CLASS AND SUBTYPE OF HYBRIDOMAS

HYBRIDOMA	ISOTYPE*
179.4	IgG_1
F47-6	$IgG_{1/3}$
F-30-20	$IgG_{1/3}$
F47-2	$IgG_{1/3}$

* Determined using commercial kit (Bio-Rad)

TABLE 3

ANTIBODY TITERS* TO OUTER MEMBRANES VS. WHOLE CELLS

	SAMPLE	
HYBRIDOMA	OUTER MEMBRANES	WHOLE CELLS
179.4	81920	40960
F47-6	40	20480
F30-20	80	20480
F47-2	80	10240

* Antibody Titer by ELISA

REFERENCE

Berdal, B.P. and Soderlund, E. 1977. Cultivation and isolation of *Francisella tularensis* on selective chocolate agar, as used routinely for the isolation of gonococci. Acta Pathol. Microbiol. Scand. Sec. B. **85**:108-109.

Boyce, J.M. 1975. Recent trends in the epidemiology of tularemia in the United States. J. Infect. Dis. **131**:197-199.

Brandt, W.E., Buescher, E.L. and Hetrick, F.M. 1967. Production and characterization of arbovirus antibody in mouse ascites fluid. Amer. J. Trop. Med. Hyg. **16**:339-347.

Bukovsky, J. and Kennett, R.H. 1987. Simple and rapid purification of monoclonal antibodies from cell culture supernatants and ascites fluids by hydroxylapatite chromatography on analytical and preparative scales. Hybridoma **6**:219-228.

Chamberlain, R.E. 1965. Evaluation of live tularemia vaccine prepared in chemically defined medium. Appl. Microbiol. **13**:232-235.

Cherwonogrodzky, J.W., Dubray, G., Moreno, E. and Mayer, H. 1990. Antigens of *Brucella*, p. 19-63 *In* K. Nielsen and J.R. Duncan (ed.) Animal Brucellosis, CRC Press, Boca Raton.

Chiewslip, D. and McCown, J.M. 1972. Elimination of repeated clot formation in mouse ascites fluid containing arbovirus antibodies. Appl. Microbiol. **24**:288-289.

Cimolai, N., Schryvers, A., Bryan, L.E. and Woods, D.E. 1988. Culture-amplified immunological detection of *Mycoplasma pneumoniae* in clincial specimens. Diagn. Microbiol. Infect. Dis.**9**:207-212.

Evans, M.E., Gregory, D.W., Schaffner, W. and McGee, Z.A. 1985. Tularemia: A 30-year experience with 88 cases. Medicine **64**: 251-269.

Francis, E. 1928. Summary of present knowledge of tularaemia. Medicine **7**:411-432.

Francis, E. and Evans, A.C. 1926. Agglutination, cross-agglutination, and agglutinin absorption in tularaemia. Public Health Rep. **41**:1273-1295.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685.

Oi, V.T. and Herzenberg, L.A. 1980. Immunoglobulin-producing hybrid cell lines, p. 351-372. *In* B.D. Mishell, and S.M. Shiigi (eds.), Selected Methods in Cellular Immunology. W.H. Freeman and Company, San Francisco.

Ormsbee, R.A., Bell, J.F. and Larson, C.A. 1954. Studies on *Bacterium tularense* antigens: 1. The isolation, purification and biologic activity of antigen preparations from *Bacterium tularense* J. Immunol. **74**:351-358.

Owen, C.R. 1970. *Francisella* infections, p. 468-483. *In* H.L. Bodily, E.L. Updyke and J.O. Mason (eds.), Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infection, 5th edition. American Public Health Association, New York.

Prochazka, O. and Dubanska, H. 1972. The antigenic structure of *Francisella tularensis* antigens: 1. Isolation of the ether antigens and its function. Folia Microbial. **17**:232-238.

Russel, P.K., Chiewsilp, D. and Brandt, W.E. 1970. Immunoprecipitation analysis of soluble complement-fixing antigens of dengue viruses. J. Immunol. **105**:838-845.

Sanders, C.V. and Hahn, R.J. 1968. Analysis of 106 cases of tularaemia. J. La. State Med. Soc. **120**:391-393.

DOCUMENT CONTROL DATA

(Security classification of title, body of abstract and indexing annotation must be entered when the overall document is classified)

1. ORIGINATOR (the name and address of the organization preparing the document. Organizations for whom the document was prepared, e.g. Establishment sponsoring a contractor's report, or tasking agency, are entered in section 8.)

Defence Research Establishment Suffield

2. SECURITY CLASSIFICATION (overall security classification of the document, including special warning terms if applicable)

UNCLASSIFIED

3. TITLE (the complete document title as indicated on the title page. Its classification should be indicated by the appropriate abbreviation (S,C,R or U) in parentheses after the title.)

Purification and Partial Characterization of Monoclonal Antibodies of *Francisella tularensis*

4. AUTHORS (Last name, first name, middle initial. If military, show rank, e.g. Doe, Maj. John E.)

Bhatti, A.R., Wong, J.P. and Woods, D.E.

5. DATE OF PUBLICATION (month and year of publication of document)

July 1992

6a. NO. OF PAGES (total containing information. Include Annexes, Appendices, etc.)

17

6b. NO. OF REFS (total cited in document)

18

6. DESCRIPTIVE NOTES (the category of the document, e.g. technical report, technical note or memorandum. If appropriate, enter the type of report, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.)

Memorandum

8. SPONSORING ACTIVITY (the name of the department project office or laboratory sponsoring the research and development. Include the address.)

9a. PROJECT OR GRANT NO. (if appropriate, the applicable research and development project or grant number under which the document was written. Please specify whether project or grant)

9b. CONTRACT NO. (if appropriate, the applicable number under which the document was written)

10a. ORIGINATOR'S DOCUMENT NUMBER (the official document number by which the document is identified by the originating activity. This number must be unique to this document.)

Suffield Memorandum No. 1383

10b. OTHER DOCUMENT NOS. (Any other numbers which may be assigned this document either by the originator or by the sponsor)

11. DOCUMENT AVAILABILITY (any limitations on further dissemination of the document, other than those imposed by security classification)

( ) Unlimited distribution
( ) Distribution limited to defence departments and defence contractors; further distribution only as approved
( ) Distribution limited to defence departments and Canadian defence contractors; further distribution only as approved
( ) Distribution limited to government departments and agencies; further distribution only as approved
( ) Distribution limited to defence departments; further distribution only as approved
( ) Other (please specify):

12. DOCUMENT ANNOUNCEMENT (any limitation to the bibliographic announcement of this document. This will normally correspond to the Document Availability (11). However, where further distribution (beyond the audience specified in 11) is possible, a wider announcement audience may be selected.)

13. ABSTRACT (a brief and factual summary of the document. It may also appear elsewhere in the body of the document itself. It is highly desirable that the abstract of classified documents be unclassified. Each paragraph of the abstract shall begin with an indication of the security classification of the information in the paragraph (unless the document itself is unclassified) represented as (S), (C), (R), or (U). It is not necessary to include here abstracts in both offical languages unless the text is bilingual).

Hybridomas secreting monoclonal antibodies against Francisella tularensis cellular antigens were isolated. These monoclonal antibodies reacted with F. tularensis in ELISA but not by immunoblot, indicating that the antibodies are directed against conformational epitopes. One of these monoclonal antibodies was directed principally against outer membrane protein (OMP) components, and the remainder are likely directed against capsular components. The OMP-specific monoclonal antibodies are F. tularensis-specific in contrast to the others which cross-react with a number of other bacterial species. These OMP-specific monoclonal antibodies are of the IgG_1 subclass, and may prove to be a useful tool for diagnosis of F. tularensis in clinical materials.

14. KEYWORDS, DESCRIPTORS or IDENTIFIERS (technically meaningful terms or short phrases that characterize a document and could be helpful in cataloguing the document. They should be selected so that no security classification is required. Identifiers, such as equipment model designation, trade name, military project code name, geographic location may also be included. If possible keywords should be selected from a published thesaurus. e.g. Thesaurus of Engineering and Scientific Terms (TEST) and that thesaurus-identified. If it is not possible to select indexing terms which are Unclassified, the classification of each should be indicated as with the title.)